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Review

Vascular Permeability Factor/Vascular Endothelial Growth Factor, Microvascular Hyperpermeability, and Angiogenesis

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Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF) and vasculotropin, is a potent, multifunctional cytokine that exerts several important and possibly independent actions on vascular endothelium.[1] VPF/VEGF was originally discovered as a tumor-secreted protein that rendered venules and small veins hyperpermeable to circulating macromolecules.[2–8] Subsequently it was learned that VPF/VEGF acts directly on cultured endothelial cells (ECs) to induce transient accumulation of cytoplasmic calcium,[9] shape change,[10] cell division,[11–19] and migration.[20] In addition, VPF/VEGF alters the pattern of EC gene expression[21,22] and induces angiogenesis *in vivo*.[11,23,24]

It is this last property, its capacity to induce angiogenesis, that has excited the greatest amount of current interest in VPF/VEGF. Angiogenesis is a complex process that involves EC division, selective degradation of vascular basement membranes and of surrounding extracellular matrix, and EC migration.[25] Directly or indirectly, VPF/VEGF has the ability to affect all of these activities. Moreover, both VPF/VEGF and its two specific EC receptors are commonly overexpressed at sites of new blood vessel growth. Taken together, VPF/VEGF has come to be recognized as an important angiogenesis factor.

Puzzling to some, however, has been the potent activity of VPF/VEGF as a vascular permeabilizing agent. Microvascular hyperpermeability has not generally been regarded as a component of angiogenesis. Therefore, it has seemed incongruous that the vascular permeabilizing effect of VPF/VEGF could be related to its pro-angiogenic activity. However, as we shall recount, there is a large body of evidence indicating that microvascular hyperpermeability regularly and perhaps invariably precedes and/or accompanies angiogenesis as it occurs in a wide variety of pathological and physiological settings. Moreover, investigations into the consequences of microvascular hyperpermeability provide a rationale for its importance in vascular neogenesis.

In this review we briefly summarize the properties and functions of VPF/VEGF and its association with angiogenesis in tumors, in non-neoplastic pathologies such as wound healing and inflammation, and in certain physiological processes. In each of these settings there is good evidence that angiogenesis is accompanied by VPF/VEGF-mediated microvascular hyperpermeability. Taken together, the data indicate that microvascular hyperpermeability regularly accompanies and likely has a mechanistic role in the induction of angiogenesis.

VPF/VEGF Structure and Functions

VPF/VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of Mr 34 to 45 kd, which, upon reduction, loses all of its biological activity and separates into major bands of 17 to 22 kD.[6–8,12,15,26–28] VPF/VEGF shares low but significant sequence homology with platelet-derived growth factor (PDGF) and closer homology with placenta growth factor.[29]

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Four different VPF/VEGF transcripts encoding polypeptides of 206, 189, 165, and 121 amino acids may be expressed by human cells.[30,31] $VPF/VEGF_{165}$ is expressed predominantly in most instances. However, all isoforms arise from alternative splicing of a single gene whose coding region is divided among eight exons; typical of a secreted protein, the DNA structure predicts a hydrophobic leader sequence that is absent from the mature protein.[30] The several different VPF/VEGF isoforms apparently express identical biological activities. However, it appears that $VPF/VEGF_{121}$ and to a large extent $VPF/VEGF_{165}$ are secreted in soluble form, whereas the two larger isoforms remain cell-associated, perhaps because of their greater affinity for cell-surface proteoglycans.[31,32] VPF/VEGF was originally purified on the basis of its affinity for heparin.[4,8] Nonetheless, the affinity of $VPF/VEGF_{165}$ for heparin is substantially lower than that of other typical heparin-binding growth factors such as basic fibroblast growth factor[4] and the smaller $VPF/VEGF_{121}$ isoform does not bind to heparin.[32]

VPF/VEGF acts directly and selectively on vascular ECs by way of two Class III receptor tyrosine kinases, flt-1 and KDR, that are expressed predominantly, if not exclusively, on vascular endothelium. Several effects follow VPF/VEGF interaction with EC receptors. The earliest to be detected is an increase up to fourfold of cytoplasmic calcium ($[Ca^{2+}]_i$); $[Ca^{2+}]_i$ begins to increase within seconds after exposure of cultured ECs to subpicomolar concentrations of VPF/VEGF, making this measurement the most sensitive assay currently available for detecting VPF/VEGF activity.[9] The effect of VPF/VEGF on EC $[Ca^{2+}]_i$ resembles that of other EC agonists such as thrombin and histamine and includes increased release of von Willebrand factor; however, as noted above, VPF/VEGF acts by way of distinct EC receptors, and its action is unaffected by inhibitors of thrombin and histamine.[9] Like other agonists that increase $[Ca^{2+}]_i$, VPF/VEGF stimulates IP_3 accumulation and is thought to act through a phosphoinositide-specific phospholipase C.[9]

VPF/VEGF was originally discovered because of its ability to increase the permeability of microvessels, primarily postcapillary venules and small veins, to circulating macromolecules. It is one of the most potent vascular permeabilizing agents known, acting at concentrations below 1 nmol/L, and, as measured in the Miles assay, with a potency some 50,000 times that of histamine.[1,2,4,10,33] VPF/VEGF acts to permeabilize a number of vascular beds, including those of the skin, peritoneal wall, mesentery, and diaphragm.[2,4,34] The vascular permeabilizing effect occurs rapidly, becoming evident within several minutes of VPF/VEGF injection.[2,4,34] Like its effects on EC $[Ca^{2+}]_i$, the vascular hyperpermeability resulting from a single injection of VPF/VEGF is transient and reversible, persisting for <30 minutes, and is not associated with detectable injury to ECs or to other microvascular components. The vascular permeabilizing action of VPF/VEGF is not blocked by antihistamines or by any of a variety of inhibitors of inflammation including those that inhibit platelet activating factor[2,4,33] (D. Senger, unpublished data).

VPF/VEGF apparently increases microvascular permeability by enhancing the functional activity of a recently described organelle, the vesicular-vacuolar organelle (VVO).[35,36] VVOs are grape-like clusters of uncoated vesicles and vacuoles deployed at intervals in the cytoplasm of ECs lining venules and small veins. The individual vesicles and vacuoles comprising VVOs are bounded by trilaminar unit membranes and are reminiscent of the 60 to 80-nm vesicles or caveolae that have been described in capillary endothelium. In contrast to caveolae, VVOs are elaborate structures, each of which, in a single section plane, comprises an average of 12 (up to as many as 20) individual vesicles or vacuoles. Commonly, VVOs span the entire thickness of EC cytoplasm from the luminal to the abluminal plasma membranes. The individual vesicles and vacuoles that make up VVOs interconnect with each other and with the EC plasma membranes by means of fenestrae that may be open or closed by diaphragms. When fenestrae are open, macromolecular tracers are able to pass between interconnecting vesicles and vacuoles, and their flow can be followed at sequential intervals across the EC cytoplasm from the vascular lumen to the abluminal basal lamina; thus, VVOs provide a pathway whereby plasma and plasma proteins may exit the circulation and enter the tissues.

In normal adult tissues, extravasation of circulating macromolecules from the microvasculature is quite limited but, to the extent that it occurs, takes place largely (eg, horseradish peroxidase) or entirely (eg, ferritin, dextrans) by way of VVOs.[35] In tumor vessels, or in normal skin following local injection of VPF/VEGF, VVO function is substantially upregulated (unpublished data). The mechanisms by which VPF/VEGF upregulates VVO function remain to be determined, but VPF/VEGF has been localized by immunocytochemistry to the abluminal plasma membrane and to VVOs of tumor vessel ECs.[36] Together these data suggest that VPF/VEGF may act to open fenestrae, thereby facilitating tracer extravasation. They also suggest that VVOs may correspond to the "large

pores" that physiologists have postulated in endothelial cells to account for the transendothelial transport of macromolecules.[37]

In addition to increasing EC cytoplasmic $[Ca^{2+}]_i$ and permeability to macromolecules, VPF/VEGF causes ECs from several different sources to assume an elongated shape and stimulates their replication.[10–19] VPF/VEGF also stimulates the migration of ECs,[20,38] mouse monocytes,[39] and fetal bovine osteoblasts.[40] To our knowledge, these last are the only examples thus far in which VPF/VEGF exerts a direct biological effect on cells other than vascular endothelium. More generally, VPF/VEGF alters the pattern of EC gene activation, upregulating the expression of both plasminogen activators (PAs), uPA, and tPA, as well as the PA inhibitor PAI-1.[21] VPF/VEGF also induces the expression of another EC protease, interstitial collagenase,[22] and tissue factor.[39] Finally, and of particular relevance to this discussion, VPF/VEGF promotes angiogenesis in a variety of assay systems, both *in vivo* and *in vitro*.[11,23,24]

Expression and Distribution of VPF/VEGF and its Receptors in Angiogenesis

VPF/VEGF Expression

VPF/VEGF is substantially overexpressed at both the mRNA and protein levels in a high percentage of malignant animal and human tumors,[2–4,41–44] as well as in many transformed cell lines.[5] Autochthonous human tumors that overexpress VPF/VEGF include a majority of carcinomas arising in the colon, stomach, pancreas, kidney, bladder, and breast, and glioblastomas; exceptions include lobular carcinomas of the breast and papillary kidney tumors that express little or no VPF/VEGF mRNA as detected by *in situ* hybridization.[42,44] Benign tumors have been less carefully studied but benign adenomatous polyps arising in the colon do not express VPF/VEGF above levels found in normal colonic mucosa.[41] However, VPF/VEGF mRNA is overexpressed by ductal carcinomas *in situ* of the breast before evidence of tumor invasion.[44]

VPF/VEGF is also overexpressed prominently in certain important non-neoplastic pathological states that, like tumors, are characterized by angiogenesis. Thus, epidermal keratinocytes, as well as a subpopulation of macrophage-like cells, overexpress VPF/VEGF mRNA in healing cutaneous wounds,[45] psoriasis,[46] and delayed-type hypersensitivity.[47] Similarly, cardiac myocytes overexpress VPF/VEGF in ischemic myocardium[48,49] as do synovial lining cells in the highly vascularized pannus of rheumatoid arthritis.[38,50]

Finally, VPF/VEGF is expressed at high levels by the placenta,[51–53] by many fetal tissues,[54,55] and by a number of normal adult tissues undergoing physiological angiogenesis; eg, in the proliferating endometrium[56] and in the corpus luteum.[57,58] VPF/VEGF is also expressed at low levels in a wide variety of normal adult human and animal tissues and at higher levels in a few select sites, namely, podocytes of the renal glomerulus, cardiac myocytes, prostatic epithelium and semen, and certain epithelial cells of the adrenal cortex and lung.[53,59–61] High VPF/VEGF expression in these last sites is exceptional in that they are not characterized by new blood vessel formation.

Recent studies have sought to determine the mechanisms regulating VPF/VEGF expression. As noted above, constitutive overexpression of VPF/VEGF has been associated with the transformed phenotype and with vascularized, autochthonous, and transplantable tumors growing *in vivo*. VPF/VEGF is also overexpressed cyclically in certain processes involving neovascularization (menstrual cycle, corpus luteum formation, etc.) that are under hormonal control. In addition, certain cytokines and growth factors stimulate VPF/VEGF mRNA expression; apparently these effects are target cell specific in that transforming growth factor-α (TGF-α), and to a lesser extent epidermal growth factor, are stimulatory in keratinocytes,[46] whereas other cytokines such as TGF-β and PDGF upregulate VPF/VEGF expression in other types of cells.[62–65] In all of these instances, VPF/VEGF overexpression is associated with angiogenesis.

Finally, tissue hypoxia has long been associated with angiogenesis, and there is good evidence that local oxygen concentrations regulate VPF/VEGF expression; in fact, the VPF/VEGF sequence shares elements with another oxygen-sensitive gene, erythropoietin.[66] In hypoxic tissues, VPF/VEGF mRNA expression is often increased above already high preexisting levels; eg, in viable tumor cells situated immediately adjacent to zones of tumor necrosis, in hypoxic myocardium, etc.[41–44,48,49,67] Moreover, in tumors with foci of relative hypoxia, VPF/VEGF mRNA may be expressed not only by malignant cells but also by stromal cells.[41,42] These findings are consistent with those of *in vitro* experiments where limiting oxygen concentration provokes a number of cell types to overexpress VPF/VEGF mRNA.[66–68]

Expression of VPF/VEGF Receptors

As noted earlier, two high-affinity VPF/VEGF receptors have been described in vascular endothelium, flt-1 and KDR; both are transmembrane proteins with cytoplasmic tyrosine kinase domains.[69–74] It is presumed, but not yet proven, that all of the effects of VPF/VEGF on vascular endothelium are mediated through one or both of these receptors.

In situ hybridization detects low level expression of both flt-1 and KDR mRNAs in the glomerular ECs of normal adult kidneys,[60] but signal is not detectable in most other normal adult tissues. However, at sites of VPF/VEGF overexpression, both flt-1 and KDR are commonly and strikingly overexpressed in adjacent microvascular ECs. Examples include various human and animal tumors,[41–44] the dermis in psoriasis[46] and contact dermatitis,[47] and the pannus of rheumatoid arthritis.[38,50] Rarely, one or another VPF/VEGF receptor has been found to be expressed by cells other than ECs where their function, if any, is unclear.[75,76]

Deposition of VPF/VEGF in Microvessels Adjacent to Sites of Its Overproduction

In addition to its detection in the cytoplasm of tumor cells, strong immunohistochemical staining for VPF/VEGF has been consistently demonstrated in adjacent hyperpermeable and replicating microvessels.[41–43,77] However, these microvessels do not themselves express detectable VPF/VEGF mRNA by *in situ* hybridization, and therefore the observed immunohistochemical staining is thought to represent binding of VPF/VEGF secreted by adjacent tumor cells. Similar microvessel staining has been observed at non-neoplastic sites of VPF/VEGF overproduction and angiogenesis; eg, in the microvessels supplying rheumatoid arthritis pannus[50] and ovarian corpora lutea.[57]

Recently, ultrastructural immunocytochemistry has localized tumor microvessel-bound VPF/VEGF more precisely to the abluminal surface of the EC plasma membrane and to VVOs.[36] This pattern of VPF/VEGF deposition could reflect binding to specific VPF/VEGF receptors. However, the intensity of the staining, the potency of VPF/VEGF action at low (sub-pmol/L to low nmol/L) concentrations, and the relatively small numbers of flt-1 and KDR receptors on individual ECs[78] (M Detmar, unpublished data) argue in favor of other possibilities, eg, binding to cell-surface proteoglycans in a manner analogous to that of basic fibroblast growth factor[79] or TGF-β.[80] Binding of VPF/VEGF to ECs could have functions other than cell signaling; eg, it may serve to keep VPF/VEGF localized, preventing the widespread dissemination of a potent and potentially deleterious biological activity. In any event, VPF/VEGF immunostaining serves to identify blood vessels at sites of VPF/VEGF overproduction, distinguishing them from vessels elsewhere in the body. This finding has raised the possibility that VPF/VEGF may provide a useful target for tumor imaging and/or therapy.[77,81]

Association Between Microvascular Hyperpermeability and Angiogenesis

The findings presented above link overexpression of VPF/VEGF (and its EC receptors) to angiogenesis as it occurs in many different pathological and physiological settings. This association makes intuitive sense in that VPF/VEGF is an angiogenic factor and as such might be expected to stimulate ECs to divide, migrate, and express matrix-degrading proteases, all properties characteristic of ECs in newly forming blood vessels. However, in addition to its other properties, VPF/VEGF renders microvessels hyperpermeable to circulating macromolecules, acting with great potency. An obvious question therefore arises: Is vascular hyperpermeability a consistent feature of angiogenesis? In fact, although rarely commented upon, the answer to this question is a categorical "yes". Published data from a number of different laboratories indicate that, wherever angiogenesis has been investigated, newly formed microvessels are hyperpermeable.

The most extensive evidence for an association between microvascular hyperpermeability and angiogenesis has come from studies of solid animal tumors. That the blood vessels supplying such tumors might be hyperpermeable was suggested some two decades ago by Gullino,[82] who found that the concentrations of plasma proteins and water in tumor stroma were substantially higher than in the stroma of corresponding normal tissues. Moreover, direct permeability measurements with a wide variety of intravenously injected macromolecular tracers have convincingly demonstrated the innate leakiness to plasma proteins of the microvessels supplying tumors.[2,3,81,83–91] Studies of ascites tumors have provided additional strong support for the inherent leakiness of tumor vessels.[4,34] In addition, they have demonstrated high concentrations of immunoreactive and bioactive VPF/VEGF in tumor ascites fluid[4,34,92] and have shown that vascular hyperpermeability precedes and accompanies angiogenesis.[34,93] In all of the tumors that we have studied, vascular leakage has occurred primarily by way of VVOs and could not

be attributed to passage of tracers through inter-EC junctions or to trivial explanations such as EC injury.[35,36,88]

It is also clear from the work of many different investigators that microvessels are hyperpermeable in pathological angiogenesis that is not associated with neoplasia, eg, in wound healing,[45,94] rheumatoid arthritis,[50] psoriasis,[46,95,96] delayed-type hypersensitivity,[97] etc. Finally, vascular hyperpermeability is also associated with the angiogenesis associated with such physiological events as corpus luteum formation in the ovary.[56,57,98–101]

Microvascular Hyperpermeability as an Early, Mechanistic Step in the Induction of Angiogenesis

The demonstrated association between microvascular hyperpermeability and angiogenesis again raises a question: Does microvascular hyperpermeability play a mechanistic role in angiogenesis? Once again the answer is affirmative. We hypothesized more than a decade ago that plasma proteins extravasate from leaky blood vessels that supply tumors and form a new, provisional extravascular matrix that permits and indeed favors the inward migration of ECs and fibroblasts.[91,102] Acting in concert, migrating ECs form new blood vessels and fibroblasts synthesize and secrete the matrix proteins, proteoglycans, and glycosaminoglycans that make up mature tumor stroma.[3,91,102–105] According to this line of thought the extracellular matrix present in normal adult tissues might be expected to limit or even prohibit mesenchymal cell migration; this is not an unreasonable premise in that EC migration would be inappropriate in a mature tissue in which new blood vessel formation is contraindicated. Therefore, if new blood vessels and supporting connective tissue are to develop in adult tissues, as, eg, in response to the increased metabolic needs of a growing tumor, native tissue stroma must first be replaced with a new matrix that supports EC and fibroblast migration.

A large body of evidence now supports this hypothesis. Plasma proteins leaking from hyperpermeable blood vessels that supply animal tumors do in fact provide a matrix that favors mesenchymal cell migration. Within a few hours of transplant, preexisting microvessels become measurably hyperpermeable to macromolecules and tumor-supplying microvessels eventually become 4 to 10-fold more permeable than their normal tissue counterparts.[2,86,88] Moreover, plasma fibrinogen that extravasates at tumor sites rapidly clots to form crosslinked fibrin.[2,3,86,106–108] This last finding indicates, moreover, that the plasma protein extravasation that tumors induce is relatively nondiscriminating in that, to form an extravascular clot, a variety of plasma clotting proteins are required and must extravasate along with fibrinogen; these include at least clotting factors V, VII, X, and XIII, and prothrombin.[109,110] The fibrin gel that is deposited in tumor stroma is itself modulated by plasmin, which is generated locally from another leaked plasma protein, plasminogen, by the action of tumor-(and perhaps EC-) secreted plasminogen activators.[2,21,86,91,102,111] Plasmin also activates matrix metalloproteases, which are capable of digesting other matrix elements. The extent of fibrin deposition and its persistence over time vary extensively among different tumors, as does the amount of mature stroma generated by different tumors. Apparently these differences depend on quantitative balances among vascular hyperpermeability, clotting, and fibrinolysis that are unique to individual tumors.[86] These balances are further complicated because, as noted earlier, one of the actions of VPF/VEGF on ECs is to stimulate the synthesis of proteins that affect both coagulation (tissue factor)[39] and fibrinolysis (uPA, tPA, and PAI-1).[21]

Supportive evidence for our hypothesis also comes from *in vitro* studies that have provided direct evidence that crosslinked fibrin of the type deposited in tumors provides a matrix that supports and favors cell migration.[112,113] Furthermore, implantation of crosslinked fibrin in guinea pigs, in the absence of tumor cells, induces the progressive ingrowth of new blood vessels and fibroblasts, which together generate vascularized stroma of the type found in many tumors.[3,91,102,103,114] Fibrin exerts its pro-angiogenic effect at least in part by providing a favorable surface for cell adhesion and migration, presumably via its arg-gly-asp (RGD) sequence. Though not yet investigated, it is very likely that other circulating RGD-containing plasma proteins also extravasate from leaky blood vessels at sites of tumor growth and there contribute to the generation of an extracellular matrix that favors angiogenesis and new stroma formation; candidate plasma proteins include fibronectin and vitronectin.

If the hypothesis we have proposed for tumor angiogenesis is valid, it might also be expected to apply to the angiogenesis that occurs in non-neoplastic settings. In fact, this seems to be the case, and we (and others) have previously called attention to the many similarities between tumor stroma generation and wound healing.[91,115] Other examples in which overexpression of VPF/VEGF and its receptors, plasma protein extravasation, and deposition of extravascu-

lar fibrin accompany angiogenesis include corpus luteum formation, psoriasis, contact allergy, and rheumatoid arthritis.[45–47,50,56,94,97,116–121] Together these findings suggest that angiogenesis, whenever it occurs in adult tissues in response to diverse stimuli, is mediated by the triggering of a common pathway, initiated in every instance by VPF/VEGF overexpression and heightened vascular permeability.

Summary and Conclusions

VPF/VEGF is a multifunctional cytokine that contributes to angiogenesis by both direct and indirect mechanisms. On the one hand, VPF/VEGF stimulates the ECs lining nearby microvessels to proliferate, to migrate, and to alter their pattern of gene expression. On the other hand, VPF/VEGF renders these same microvascular ECs hyperpermeable so that they spill plasma proteins into the extravascular space, leading to the clotting of extravasated fibrinogen with deposition of a fibrin gel. Extravascular fibrin serves as a provisional matrix that favors and supports the ingrowth of new blood vessels and other mesenchymal cells that generate mature, vascularized stroma. These same principles apply in tumors, in several examples of non-neoplastic pathology, and in physiological processes that involve angiogenesis and new stroma generation. In all of these examples, microvascular hyperpermeability and the introduction of a provisional, plasma-derived matrix precede and accompany the onset of EC division and new blood vessel formation. It would seem, therefore, that tumors have "borrowed" fundamental mechanisms that developed in multicellular organisms for purposes of tissue defense, renewal, and repair.

VPF/VEGF, therefore, has taught us something new about angiogenesis; namely, that vascular hyperpermeability and consequent plasma protein extravasation are important, perhaps essential, elements in its generation. However, this finding raises a paradox. While VPF/VEGF induces vascular hyperpermeability, other potent angiogenic factors apparently do not, at least in subtoxic concentrations that are more than sufficient to induce angiogenesis.[11] Nonetheless, wherever angiogenesis has been studied, the newly generated vessels have been found to be hyperpermeable. How, therefore, do angiogenic factors other than VPF/VEGF lead to the formation of new and leaky blood vessels? We do not as yet have a complete answer to this question. One possibility is that at least some angiogenic factors mediate their effect by inducing or stimulating the expression of VPF/VEGF. In fact, there is already one clear example of this. TGF-α is a potent angiogenic factor but does not itself increase microvascular permeability. However, TGF-α strikingly upregulates VPF/VEGF expression in cultured keratinocytes and is thought to be responsible, at least in part, for the overexpression of VPF/VEGF in psoriasis.[46] Moreover, overexpression of TGF-α, along with that of the EGF receptor with which it interacts, is characteristic of many malignant tumors, raising the possibility that TGF-α acts to stimulate VPF/VEGF expression in other types of epithelial cells and in this manner induces angiogenesis. Further studies that elucidate the crosstalk among various angiogenic factors are likely to contribute importantly to a better understanding of the mechanisms by which new blood vessels are formed in health and in disease.

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